Delivering siRNA with Dendrimers: In Vivo Applications

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ABSTRACT

Background: Over the last decades, gene therapy has emerged as a pioneering therapeutic approach to treat or prevent several diseases. Among the explored strategies, the short-term silencing of protein coding genes mediated by siRNAs has a good therapeutic potential in a clinical setting.

Material & Methods: However, the widespread use of siRNA will require the development of clinically suitable, safe and effective vehicles with the ability to complex and deliver siRNA into target cells with minimal toxicity. Lately, dendrimers have gained considerable attention as non-viral vectors in nucleic acid delivery due to their unique structural characteristics (globular, well defined and highly branched structure, multivalency, low polydispersity and tunable nanosize), along with their relevant capacity to complex and protect nucleic acids in compact nanostructures, which can be functionalized with targeting moieties in order to get cell specificity.

Conclusion: Here, we present an overview of the state-of-the-art of the most significant and recent advances on the use of dendrimers as siRNA delivery vectors, with particular focus on the in vivo applications. We will cover the use of different dendrimers, distinct administration routes, toxicity issues, as well as the target tissue or disease, highlighting the potential of dendrimers as nanocarriers for therapeutic and biomedical applications.

Keywords: Dendrimers, RNAi, siRNA, Gene therapy, In vivo, Nanomedicine, Nanoparticles, Biodistribution.

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1. INTRODUCTION

1.1. RNA Interference (RNAi)

In 1998, an unforeseen gene-silencing event in nematodes [1], later called RNA interference (RNAi), was discovered. Further investigations confirmed that a similar response also existed in mammalian cells [2]. Briefly, RNAi is a post-transcriptional regulatory mechanism that consists of the cleavage, by an endonuclease called Dicer [3], of endogenous long double stranded RNA (dsRNA) molecules into small interfering RNAs of about 21–23 nucleotides that are incorporated into the RNA-Induced Silencing Complex (RISC). After, RNA helicases unwind the double-stranded siRNA and the antisense strand guides RISC to the complementary messenger RNA (mRNA) for degradation. RNAi can be mimicked by synthetic RNA molecules, such as micro RNA (miRNA), short hairpin RNA (shRNA) and small interference RNA (siRNA). The latter mediates gene silencing by inducing sequence-specific cleavage of targeted mRNA in a catalytic process [4-6] (Fig. 1).

Quickly, it was appreciated the potential of RNAi-based therapies for down-regulating abnormal gene expression or mutated proteins involved in some human diseases that are not targetable by conventional drugs. Among the strategies for turning off protein expression, RNAi mediated by synthetic siRNAs has a good potential in a clinical setting for the exploration of new therapeutics. In fact, several studies have already stressed the potency of siRNA-based strategies in knocking down the expression of specific targets in vivo for the treatment of different diseases [7-10], and many proposed therapies have completed phase I clinical trials, while others have already progressed into more advanced evaluation stages [11-13].

1.2. Advantages of siRNA Therapeutics

siRNA can be categorized into conventional, sticky and Dicer substrate siRNA. Conventional siRNA is synthetic dsRNA molecule bearing 19-25 base pairs and two dTdT overhangs at the 3′-ends, which is designed to mimic the Dicer cleavage product and, therefore, directly enter RISC for triggering the RNAi mechanism [14]. Different from conventional, siRNA with short complementary An/Tn (n = 5–8) overhangs, (instead of dTdT overhangs), named sticky siRNA, can be used, which considerably increases gene silencing efficiency. In addition to sticky siRNA, competent Dicer substrate siRNA (dsiRNA) of 25–30 nucleotides can be up to 100-fold more potent than conventional 21-mer duplex siRNA when targeted to the same sequence location. This dsiRNA bears an asymmetric blunt end and an over-hang at the 3′-end that can be optimally processed by Dicer, hence yielding higher and more durable RNAi effect.

Among the different mechanisms to silence protein expression, siRNAs area very good choice as it is easy to dis-cover unmodified siRNAs that work with high potency. Compared to antisense oligonucleotides (ASOs), siRNAs have two strands, which simplifies their delivery and make siRNAs more resistant to nuclease degradation [15-17]. In addition, siRNAs show a more potent and prolonged therapeutic effect, thanks to the mentioned catalytic mechanism. Regarding dsRNAs,the antisense strand of siRNA is completely complementary to the mRNA target and has higher target recognition and binding compared to the other RNA molecules which are only partially complementary to the target mRNA [18, 19]. Moreover, the total understanding of the methodology for siRNA synthesis enables preparation of a siRNA that selectively shuts off a targeted gene. In fact, specific siRNAs are now commercially available to silence almost any gene and in various different organisms. Important to notice that the down-regulation using siRNAs is transient. This may be adequate for some purposes where a temporary effect is needed. In situations requiring a persistent effect, repeated administrations are necessary.

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Due to the high power and lower off-target interaction of siRNA among all the antisense molecules, the use of siRNA has been considered the most promising tool being applied to personalized medicine [20, 21].

1.3. siRNA: Barriers, Challenges and Delivery Vectors

Despite the great advances achieved during the last years on the understanding and harnessing of the RNAi-mediated gene silencing mechanisms, some aspects are yet to be improved in order to translate RNAi-based therapeutics into the clinics [22-24]. For instance, few studies reported non-specific effects triggered by siRNA-based therapeutics, as the mentioned off-target silencing (although low) and activation of the interferon system [25-29]. The recognition of this unintended gene modulation has led to the improved design of siRNA sequences by the use of appropriate algorithms, the establishment of several rules for siRNA design and the introduction of chemical modifications into the nucleic acid (NA) structure, which ultimately lead to minimal off-target activity [30].

Nevertheless, the main challenge is related to the siRNA transport and delivery. Due to its large molecular weight (~14 kDa in average) and polyanionic nature (negative phosphate groups), naked siRNA does not freely cross the cell membrane. Also, naked siRNA can be rapidly degraded by endonucleases when administrated and has a very short life in circulation. Therefore, it is now commonly accepted that the widespread use of siRNA therapeutics will require the use of clinically suitable, safe and effective delivery vehicles with the ability to protect naked siRNA, surpass the different extra- and intracellular barriers and efficiently deliver it into cells with minimal toxicity [31-36]. The extra- and intracellular barriers and the corresponding challenges in the vector-mediated siRNA delivery include: a) protect siRNA against degradation by endonucleases present in the extra- and intra-cellular milieu, b) avoid renal clearance, particularly relevant in the case of systemic administration, c) delivery to the desired site of action and avoid non-specific delivery, d) pro-mote cellular internalization, e) favor endosomal escape, f) siRNA release from the vector and access to the cytoplasmic and to the RNAi machinery to permit an effective gene silencing process, and g) avoid vector intra- and extracellular accumulation by ineffective clearance after accomplishing their biological function (i.e. release the siRNA cargo). Additionally, the delivery vector must avoid unspecific binding to serum proteins, preventing aggregation and recognition by the complement system.

Two classes of vehicles for siRNA are clearly distinguishable: viral and non-viral based vectors [37-39]. Even though viral based vectors have been extensively studied, showing high transfection efficiency rates, the adverse effects such as mutagenesis and immunogenicity are important obstacles still to overcome [40-43] which have limited their use in vivo. Furthermore, the low-scale production and storage difficulties [43] remain crucial limitations that raise concerns for its translation to the clinic. These facts have encouraged the development of non-viral cationic vehicles for siRNA delivery [39].

Electrostatic interactions between negatively charged siRNA and cationic polymers or macromolecules are the basis, in the great majority of cases, for the non-viral vector-mediated siRNA delivery. Most of the siRNA carriers tested so far have been mainly based on cationic systems previously developed for the delivery of plasmid DNA (pDNA) such as liposomes, lipids, cell penetrating peptides, natural and synthetic polymers and, more recently, dendrimers. However, fundamental differences between pDNA and siRNA molecules regarding size, morphology, flexibility and charge, can result in a less efficient interaction and lower protection of the latter [44]. Thus, the cationic vectors used for gene delivery do not necessarily result in optimal vectors for this small double-stranded NA [45-47]. Consequently, design and optimization of cationic vectors for the delivery of siRNA are required [47].

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2. DENDRIMERS

In 1978, Voëgtle et al. synthesized a new class of multiarmed molecules ("cascade molecules") [48]. The further increase in the complexity of these branched molecules by Denkewalter, Tomalia, Newkome, Frechet and colleagues led to larger structures/architectures, then coined as "dendrimers" [49-52]. Dendrimers comprise: a) a central core with at least two reactive groups, b) repeating units linked to the central core and distributed/arranged in concentric layers named "generations" (G), and c) high number of terminal functional groups on their surface (Fig. 2a). This "treelike" structure is depicted by the word dendrimer (from Greek: dendron = tree and meros = part), which relates to the distinctive organization of their branched blocks.

Soon, it was shown that dendrimers, in their cationic form, had something special to offer also as non-viral vectors in the gene therapy field, due to their capacity to complex and protect NAs in compact nanostructures (Fig. 2b) [53]. Moreover, their exclusive structural characteristics: globular, well-defined and highly branched structure, low polydispersity, tunable size and the possibility of controlled multifunctionalization (due to the presence of chemical handles on the dendritic periphery – multivalency), further allow a specific, fine and precise design of smart dendritic carriers for the NA delivery field.

Dendrimers are synthesized mainly by two different iterative synthetic approaches: divergent [48, 50, 51] or convergent [52], which allow their accurate design. In the divergent route, introduced by Tomalia, Newkome, and Voëgtle, dendrimers grow layer-by-layer from the core towards the periphery. The core molecule reacts with monomeric molecules (repeating unit) containing one reactive and at least two inactive branched sites, yielding the first-generation dendrimer (G1). Then, the surface of this G1 is activated for reaction with more repeating units. This process is repeated several times until the dendrimer of the desired generation and, therefore, size is formed. On the other hand, the convergent approach introduced by Fréchet et al. consists of the synthesis of branched individual dendrons, which are finally linked with a multifunctional core. The divergent methodology is susceptible to defective monomeric molecule assembly due to the higher number of reactions performed simultaneously, and thus, purification is usually required after each step [54]. Consequently, the probability of byproduct formation boosts with the generation, rendering lower over-all yields. Conversely, in the convergent synthesis only a limited number of groups are active per reaction, therefore, the probability of structural flaws is lower [54]. However, one disadvantage of the latter is its low ability to grow dendrimers until higher generations due to the steric hindrance between dendrons in the last step, although the selection of a suitable core (size and multivalency) can help to reduce these steric hindrances [54].

Finally, it is worth mentioning that new user- and environmentally-friendly synthetic approaches with higher reaction efficiency and step number reduction, such as "Lego" [55] and "click" [56] chemistry, and other accelerated and orthogonal synthesis strategies [57, 58], have been reported to overcome the drawbacks and the tedious purification intensive iterative processes of the previously mentioned classical strategies.

3. DESIGNING DENDRIMERS FOR siRNA DELIVERY

Cationic dendrimers represent particularly attractive class of non-viral vehicles for siRNA, since they are able to complex and protect this NA in compact nanostructures, coined "dendriplexes" (dendrimer-NA complexes) (Fig. 2b), There-fore, they contribute to surpass the previously mentioned extra- and intracellular barriers, towards an efficient delivery of exogenous therapeutic siRNA into cells.

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The development and optimization of highly efficient and rapid synthetic approaches together with the design of novel orthogonal building blocks have allowed the synthesis of an ample range of different dendritic compounds and families with monodisperse nanosizes and great number of functional end groups, what also allows attaching different ligands in a controllable manner, leading to optimized dendritic nanocarriers. Probably, the dendritic structures most extensively investigated for siRNA delivery have been the poly(amidoamine)(PAMAM) [59], poly(propylene imine) (PPI) [60] and poly(L-lysine)-based (PLL) [61, 62] dendrimers (all commercially available), as well as carbosilane [63]and triazine [47] dendrimers. Even commercial gene transfection reagents such as PolyFect®, SuperFect® and PrioFect® based on PAMAM dendrimers initially developed for DNA delivery, have also been shown to efficiently mediate siRNA delivery [64, 65].

The primary interaction between cationic dendrimers and siRNA is electrostatic. Consequently, dendriplex stability and NA delivery profile usually improve with the dendritic generation [53, 66-68]. Nevertheless, it has also been observed that higher generations do not always lead to higher transfection efficiencies [60, 69]. This could be explained since dendrimers of lower generation may interact with NAs more efficiently than the higher generations as a result of their more flexible structure [70].

Despite the charge neutralization during the condensation process, when the molar ratio between the cationic groups from the dendrimer and the phosphate groups from the NA is above 1, the complexes preserve an excess of positive charge on their surfaces, which can result in cellular toxicity. Therefore, it is important to assess not only the toxicity of the free used cationic dendrimers, but also the one that may be elicited by the dendriplexes. The latter will be more important in the short-term, i.e. during transport, whereas free dendrimer toxicity may have a long-term effect, after NA delivery upon dendriplex disassembly.

Although the internal dendrimer structure plays a significant role in determining the biological performance of these structures [71], the characteristics of their surface groups will predominantly determine pharmacokinetics and biocompatibility [72, 73]. In fact, cationic dendrimers will interact more strongly with the negatively charged surface of the cell membranes than anionic or neutral ones, and therefore they will be more cytotoxic [72, 74]. It seems clear that higher generation dendrimers, besides less biocompatible, will be more toxic because of the higher number of positive charges. Therefore, it could be concluded that cationic dendrimers toxicity is concentration and generation dependent [75, 76]. But contradictory data can be found in the literature regarding in vivo tests. Some studies show that PAMAM and PPI dendrimers, especially at low generations, are not as toxic as initially described [77-81]. However, others have reported toxicity profiles for the same dendrimers [82-84]. It should be noted that in vivo cytotoxicity will also depend on the dose and administration route [72]. Therefore, dendrimer characteristics (chemistry, size and charge – the last two ones closely related to generation) will also be important in the choice of the administration route and, consequently, on the in vivo biodistribution [72, 85-87]. These issues will be further discussed in detail in the following sections.

Because of this controversial toxicity related to cationic dendrimers, much effort has been put into decrease /improve/optimize their cytotoxicity profile by surface engineering. For instance, acetylation of PAMAM dendrimers to reduce the number of primary amines [88] or also the use of hydroxyl-terminated PAMAM G4 dendrimers with quaternized internal amines [89] are some of the investigated strategies to improve biocompatibility. However, in this regard, the most common approach for masking the dendrimers' charge and improving their solubility and biocompatibility, while increasing their circulation time in the blood stream, is to tether chains of poly(ethylene glycol) (PEG) to the dendritic backbone (PEGylation) [90] (Fig. 2). Moreover, other promising antibiofouling/non-fouling polymers have emerged as possible alternatives to PEG, such as zwitterionic polymers [91, 92], poly(2-oxazoline)s [91, 93, 94], poly peptoids [91], polycarbonates

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[95], among others. Although there are already a few reports in which some of these alternative polymers are attached to dendritic structures [92, 94], further studies are expected to test their favorable properties when linked to dendrimers.

Besides this, the possibility of precise chemical multi-decoration of dendrimers allows decreasing their unfavorable properties, such as the mentioned cytotoxicity, while improving the gene silencing effect, favoring the design of effective dendrimer-based siRNA nanocarriers. The main modification strategies of the dendrimers being explored to optimize their vectorization capability include:

1) The tethering to the dendrimer surface of different molecules, such as amino acids [47, 96-98], cyclodextrins [99-103] and hydrophobic moieties [47, 88, 104-107] in an optimized percentage and form. These modifications aim to increase the dendriplex stability and siRNA protection capability, as well as to enable the parent dendrimer to traverse the cell membrane, disrupt the endosomal mem-brane and/or facilitate endosomal escape – one critical challenge when talking about non-viral vector mediated siRNA delivery [108].

2) Adjustment of the core structure [66, 68, 109-115] to enhance siRNA transfection efficiency. For instance, PAMAM dendrimers were studied with different types of core: a flexible core consisting on triethanolamine (TEA) [68, 113, 114], and a rigid poly(phenylene vinylene) (PPV) core [112]. Also, the focal point of dendrons can be used for linking moieties, mainly hydrophobic molecules, with the same aim of improving gene silencing [116, 117].Fig. (2). Schematic representation of: a) a dendrimer with attached chains of poly(ethylene glycol) (PEG) and a target molecule, b) siRNA/dendrimer complex (dendriplex).

3) Finally, the conjugation of several ligands and target molecules (Fig. 2), such as peptides and proteins, tailored to attain an efficient and site-specific (cells and tissues) siRNA delivery is also an attractive strategy [62, 103, 118-123].

In addition to these dendrimer modifications, the suitable choice of the generation number (according to the dendritic family), will also have a significant impact on the enhancement of the transfection efficiency 47, 68].

Despite all these improvements on the design of dendrimers to act as vectors for siRNA delivery, the non-biodegradability under physiological conditions of the most commonly used dendritic structures in the NA delivery field remains a weak point that scientists need to further address [54, 72, 124, 125]. After accomplishing their biological function – release the siRNA cargo – non-biodegradable dendrimers can also lead to cytotoxicity induced by the accumulation of non-degradable synthetic materials inside cells/tissues [86, 124]. Therefore, the ideal delivery vehicle should be biodegradable to prevent bioaccumulation and subsequent cytotoxicity [86, 124-126]. Moreover, the dendrimers' biodegradability can also contribute to the efficient release of the transported NA [125, 127], and consequently yield higher RNAi.

In the following sections, some of the most significant and recent advances in the use of dendrimers as siRNA vectors in in vivo applications are revised. We cover the use of different dendrimers, distinct administration routes, toxicity issues, as well as the targeting of a specific tissue or disease, highlighting the caveats and potential of dendrimers as nanocarriers for therapeutic applications.

4. IN VIVO APPLICATIONS

The administration of siRNA dendriplexes to organisms requires preliminary extensive studies in the test tube and also with cells in culture. The strategies tested in vivo are, therefore, the result of innumerous iterative improvements previously tested in vitro. The delivery of siRNA mediated by

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dendrimers has been applied to date in different paradigms, with different aims and using different strategies. These studies in vivo using dendrimers to carry siRNA will be analyzed regarding its success rate, efficiency, toxicity and organ/cell specificity.

4.1. Dendrimer Types Applied In Vivo

Among the different dendrimers explored for siRNA delivery (PAMAM, PPI, PLL, carbosilane, and triazine), PAMAM has been the most widely used and studied vector in in vivo applications (Table 1). A large variety of modifications have been performed and tested in order to overcome the known obstacles faced in the complex in vivo environment.

In 2009, an approach using polyvalent dendrimer-bearing magnetic nanoparticles ("dendriworm"), which allow to locate in vivo the nanoparticles by imaging techniques, were used to carry siRNA to the most common and most aggressive brain cancer - a glioblastoma induced in mice [111], the most common and most aggressive brain cancer. For dendriworm development, cystamine core G4 PAMAM dendrimers were reduced yielding thiol dendrons. Then, amine-modified, cross-linked strings of spherical iron oxide nanoparticles coated with a biocompatible polymer (Dextran), named "nanoworms" [128], were prepared. Finally, reduced dendrons and nanoworms were conjugated using the heterobifuctional linker succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The effects of siRNA delivered by den-driworms to the brain parenchyma were studied in healthy and brain tumor tissue, after intracranial infusion with an osmotic pump for a long period of time (3 and 7 days). These dendriworms showed improved proton sponge effect and enhanced endosomal escape efficiency when compared to dendrimers or nanoworms, resulting therefore in a better siRNA silencing.

Another group has reported promising results using a flexible TEA-core PAMAM dendrimer for delivering a cocktail of anti-HIV-1 Dicer substrate siRNAs in a humanized mouse model [113]. These dendriplexes suppressed viral infection by several orders of magnitude compared to free siRNA, which constitutes a big step in the treatment of HIV infection.

Liu and colleagues developed arginine-terminated dendrimers with the aim of combining the appropriate properties of the TEA-core PAMAM dendrimer in siRNA delivery and the cell-penetrating advantages of the arginine-rich motif, which enhances cell membrane penetration. This proved to be effective in delivering siRNAs, leading to potent gene silencing in vivo [129].

An efficient strategy, also using a TEA-core PAMAM dendrimer, to deliver heat shock transcription factor 27 (Hsp27) sticky siRNA to prostate cancer cells was explored by Peng's group. It produced potent gene silencing of Hsp27, which in turn lead to a notable anticancer effect [114]. The same team also reported a lipid/dendrimer hybrid bearing a hydrophobic long alkyl chain and a low generation hydrophilic PAMAM dendron was used in a prostate cancer mouse model [130]. This system displayed the advantageous delivery features of both lipid and polymer vectors. Hsp27 siRNA was delivered locally to the tumor and a high rate of success for gene silencing and anticancer activity was obtained.

As previously mentioned, other types of dendrimers appropriately modified for in vivo applications, have also been successfully employed with relevance (Table 1). Taratula et al. proposed a strategy using PPI dendrimers in which siRNA nanoparticles were caged with a dithiol containing cross-linker molecules and coated with PEG polymer [121]. These modifications provided lateral and steric stability to withstand the aggressive environment in the blood. Reduction of the intramolecular disulfide bond in the cytoplasm triggered the release of siRNA in cancer cells with success. The targeting delivery approach was obtained by conjugation to the dendriplexes of the luteinizing hormone-releasing hormone peptide, which directed the nanoparticles to the lung cancer cells.

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Serramia et al. reported the delivery of siRNA to the brain using a carbosilane dendrimer carrier [131]. After cell culture studies with these dendriplexes, their ability to cross the blood–brain barrier (BBB) was investigated and it was found that they efficiently reached the brain after a retro-orbital injection administration.

Very recently, 1,500 modular dendrimers based on 2-(acryloyloxy)ethyl methacrylate (AEMA), with chemically diversified cores, peripheries and generations modified with ester bonds, were synthesized by a sequential modular synthesis strategy [87]. From these, 50 top lead G1 to G4 dendrimers were tested in vivo for siRNA delivery. After selecting five dendrimers that led to knockdown with high potency, toxicity studies were performed and three dendrimers were excluded due to significant toxicity, which was shown by weight loss. The remaining two best dendrimers were tested in an aggressive liver cancer model, where the one with a lineal pentamine core and octane-1-thiol surface modified, was the best tolerated and led to a significant high siRNA accumulation in the tumor.

4.2. Route of Administration

Therapeutics can be introduced into the body by various routes such as intravenous (i.v.), intraperitoneal (i.p.), intra-muscular, subcutaneous, among others. [132]. The route of administration has a profound effect on the accessibility of molecules/structures to their respective target site, as well as the speed and the efficiency with which the therapeutic will act. Consequently, it will have a profound impact on the in vivo performance of the administered system. When choosing the route of administration for the nanoparticle/dendriplex, one has to equate various aspects: a) the accessibility to the target site, b) the least invasive technique that still is effective in delivering therapeutic amounts of the selected agent, c) the volumes and frequency of administration, d) possible side effects, e) stability of the dendriplex after delivery, f) clearance through the body, and g) toxicity.

The most traditional route for the delivery of dendrimers and dendriplexes is the i.v. route [123 and Table 1]. With this systemic administration, it is common to find dendriplexes in the liver as well as in the spleen (though not so often studied). This accumulation is explained since these are organs of the reticuloendothelial system responsible for the clearance of foreign materials by macrophage uptake. It is also common to find dendriplexes in the kidneys. Renal excretion of dendrimers after systemic administration is known and therefore it is expected that dendrimers are present in this organ. For example, the fate of free cationic PAMAM dendrimers (G3 and G4) and anionic PAMAM dendrimer (G 2.5, 3.5 and 5.5) after i.v. administration has been studied, to find that these materials accumulate in the liver and also that they are cleared from the blood via the kidney though with different clearance rates [146]. Moreover, it was also observed that after i.p. injection, most of the administrated dendrimers were transferred into the blood within an hour and exhibited a similar clearance pattern to the results found after i.v. injection. One of the main problems in systemic administration of cationic macromolecules is vector aggregation in the presence of serum that in the context of siRNA delivery mediated by dendrimers can result in nanoparticle disintegration, siRNA release and degradation. As mentioned before, PEGylation of the nanoparticle structures can prevent particle aggregation and increase their stability in serum. Therefore, this is a very common dendrimer and dendriplex modification for in vivo purposes. To overcome some of the limitations associated with systemic administration, local delivery of dendriplexes can be performed which increases site direct activity and minimizes systemic toxicity. This type of administration is found in various cancer studies (Table 1) where for example TEA-core PAMAM dendrimers complexed with siRNA have been directly administered to the tumor site [115]. Also regarding cancer, a useful strategy in brain is the use of convection enhanced delivery (CED) though it is very invasive due to the intracerebral injection [111]. A heart local injection has also been effective in improving cardiac function in a mice model of cardiovascular disease [137].

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The choice of the route of administration of the dendriplexes sometimes requires adjustments in its formulation. Such was the case in the study performed with radiolabeled [32P]-siRNA/PAMAM dendriplexes, where intranasal administration was chosen in order to reach the brain [135]. The dendriplexes were administered within an in situ forming mucoadhesive gel so as to increase the retention time of the dendriplexes in the nose mucosa and potentiate the rate of success after administration.

When repeated administrations are required due to the siRNA transient effect, more invasive routes of administration like the intracranial or intramyocardial are not a viable option.

Therefore, the choice for the route of administration has to take into account different factors, such as the purpose of the delivery, the dendrimer characteristics and the frequency of administration, which in turn will dictate the effectiveness of the procedure.

4.3. Biodistribution

As seen so far, dendrimers can be better suited for delivering siRNA to a specific organ, cell population or a diseased site depending on its composition, generation, size, surface functional groups and modifications. All of these characteristics will affect their biodistribution and, consequently, the RNAi efficiency. As an example, Anderson's group, employed a combinatorial approach, with PAMAM and PPI dendrimers modified with epoxide-terminated alkyl chains of increasing length to deliver siRNA to endothelial lung cells [107]. It was observed that the optimized formulations that included cholesterol, improved endothelial cell uptake of siRNA in vivo, consequently increasing tunica interna endothelial cell kinase (Tie2) lung knockdown. It was reasoned that when coupled with cholesterol, more serum proteins as for example albumin, were able to adsorb to the dendriplex, which in turn enhanced endothelial cell uptake. Moreover, the endothelium of other organs was also examined, but the knockdown effect was most potent in the lung because of thedendriplexes higher avidity for lung endothelial cells. These findings may have applications in the treatment of dysfunctional lung endothelium. Again, the same team synthetized a library of different dendrimers to deliver siRNA preferentially to: a) endothelial cells in the liver, or b) endothelial cells and hepatocytes, or c) endothelial cells, hepatocytes, and liver tumor cells in vivo[106]. The differential tropism of the dendriplexes to different cells within the liver was influenced by the carbon chain length of the alkyl epoxide and the ratio of cholesterol used in its formulation. Therefore, besides performing modifications to direct the dendriplexes to a specific organ, it is also possible to target the dendriplexes to distinct cell sub-populations within the same organ by tuning its formulation. Regarding cancer research, previous studies suggested that vascularized tumors experience increased uptake of nanoparticles due to the their leaky vasculature and consequent enhanced permeation and retention (EPR) effect [147]. This phenomenon is the rationale for the higher accumulation of dendriplexes after systemic administration in such tumors. To increase the amount of dendriplexes reaching the tumor (or in fact any target organ) after an i.v. administration, it is important to extend its circulating time in blood, which can be attained by, for example, PEGylation as previously mentioned [90]. Also, the size is an important parameter to take into account. Addition of successive layers (generations) gradually increases molecular size and amplifies the number of surface groups present, which in principle will allow to increase proportionally the amount of bound siRNA. But the size will also impact on the dendrimer biodistribution, as was seen using a 3D multicellular tumor spheroid (MCTS) model where smaller PAMAM dendrimers not only diffused more rapidly in the extracellular matrix, but also efficiently penetrated to the MCTS core compared to their larger counterparts [148].

In the field of nanoparticle delivery to the central nervous system (CNS), there are additional major obstacles to the success of the procedure. The BBB prevents most small molecules and nearly all macromolecules from entering the CNS [149]. Current strategies used for dendriplex delivery to the

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brain include invasive and local delivery. Direct injection is mostly appropriate for local activity, as for example in brain tumors when there are no metastases, but it is not adequate when the pathological impact is widespread and the invasiveness nature of the technique surpasses its benefits. Therefore, systemic delivery is a possible option to reach the brain when the BBB is compromised such as in stroke. However, this method of delivery also leads to nanoparticles delivery to other organs besides the brain, which might result in unintended effects. To overcome this lack of specificity, tageting moieties can be added to the surface of the dendrimer as mentioned in previous sections. This should lead to a certain preferential localization. As an example, transferrin addition to a G_3 PPI dendrimer increased significantly the dendrimer BBB crossing that in turn increased the amount in the brain [150]. Many reports also exist in cancer research where, for instance, folic acid is added to the dendrimer resulting in the targeting to the tumor [101].

To conclude, dendriplexes biodistribution depends not only on the administration site, but also on the dendrimer itself i.e. its size, composition and post-synthesis surface modifications (PEGylation, targeting molecules, etc.).

4.4. In Vivo Toxicity

Cell culture studies have shown that cationic dendrimer and dendriplex cytotoxicity is mainly attributed to the interaction of their surface positive charge with negatively charged biological membranes. The interaction of dendrimers with biological membranes can result in membrane disruption via "nanohole" formation, membrane thinning and erosion, which lead to necrosis/non-apoptotic cell death [151]. Several reports have carefully described the influence of dendrimer chemistry, size and charge on biological membranes integrity [146]. Moreover, recent works propose that, besides membrane destabilization, toxicity may also come up from impaired oxidative metabolism resulting from mitochondrial dysfunction [152] and changes in endogenous gene expression [153] that ultimately lead to apoptotic cell death.

To minimize the possible toxicity, different strategies in the synthesis and modification of the cationic dendrimers have been performed, as described above. However, to date there is no "standard" strategy in the dendrimers synthesis/modification in order to overcome a possible toxicity after in vivo administration. Some authors claim that the use of lower generation dendrimers may also diminish the likelihood of immune system activation and inflammatory response associated with the use of higher generation dendrimers [151]. But there is no consensus on this subject. For instance, Okuda and colleagues concluded that G6-PAMAM dendrimers did not induce any measurable hepatic damages [154], while a lower generation G_4-PAMAM dendrimer even induced anti-inflammatory activity [78]. Regarding surface chemical engineering, toxicity profiles are not always improved by functionalization. As an example, Albertazzi and co-workers explored the effect of unmodified and C12-acetylated G_4-PAMAM dendrimers in the CNS and they found in vivo toxicity only for the modified dendrimer [155]. However, this could be surpassed if modifications are tuned regarding aliphatic chain length and percentage of functionalization. Conflicting data can arise because each dendrimer will have its own requirements for synthesis and/or modification in order to overcome toxicity and, as commented in the previous sections, the needed modifications have to be carried out in an optimized form. Also, each intended purpose or function attributed to the dendrimer will dictate its structure, the concentration or amount needed and, concomitantly, this will affect toxicity. Thus, each dendrimer has to be evaluated in the proper context and taking into consideration the efficiency in which it reaches a certain tissue. So, in one situation the dendrimer may not be toxic while in another it can constitute a problem if for instance it accumulates in increased amounts.

Regarding dendriplexes, it is obvious that their toxicity depends on the toxicity of the parent dendrimer. However, after complexation with the NA, the toxicity, for both systems (parent

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dendrimer and dendriplex) should be equally assessed. Nevertheless, most of the reports show similar profiles, as recently reported by Chen et al., who used self-assembled small amphiphilic dendrons into nanosized supramolecular dendrimer micelles resembling high generation dendrimers [156]. These supramolecular structures showed no toxicity either alone or when complexed with the siRNA, and the purpose of effective gene silencing was achieved.It is intuitive to accept that the dose and frequency of administration are also parameters to take into account when considering toxicity after dendriplex administration [151]. A too high dose or multiple administrations with short intervals can induce, for example, a lysosomal storage problem if the dendrimers do not degrade and accumulate in this organelle [157]. A balance should then exist between the lowest amounts of administered dendriplexes with the highest possible effect elicited by them.

The best practices in this area of research indicate that toxicity in in vitro cell culture should be assessed before in vivo toxicity studies are performed, in order to prove the safety of any nanoparticle. But, even though numerous in vitro studies have shown severe undesirable effects, recent reports indicate that these cannot be always extrapolated to in vivo settings [158]. So, dendrimer and dendriplex toxicity evaluation in vivo is essential and can be performed in many ways, as for example monitoring weight loss, organ histopathology, hemolysis (red blood cell morphology), alterations in transaminases, alterations in urea nitrogen levels, behavioral studies, lactate dehydrogenase activity in serum, interferon response or inflammatory response.

Watanabe and colleagues showed that delivery of apolipoprotein B (ApoB) - specific siRNA with G6 PLL dendrimers led to knockdown of ApoB in healthy mice liver without hepatotoxicity [143]. The potential toxicity was assessed by measuring the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are two enzymes located in liver cells that leak into the circulation when liver cells are injured.

Another study intended to deliver siRNA to the lung, showed that, at high doses, the G1 dendrimer–lipid derivatives of PAMAM and PPI dendrimers used did not cause chronic increases in pro-inflammatory cytokines and animals did not suffer weight loss due to toxicity [107].

Different kinds of carbosilane-based dendriplexes have shown good toxicity profiles in peripheral blood mononuclear cells and erythrocytes over extended periods of time [159].

Fortunately, in general, surface and/or core modified cationic dendrimers as well as their siRNA/dendriplexes display a good toxicity profile, do not show major histopathological changes nor exacerbate the inflammatory response (Table 1), which is significant for further translational studies.

4.5. Therapeutic and Biomedical Applications

siRNAs can be therapeutically applied as antiviral or anticancer agents, in CNS therapeutics, in inflammation or cardiovascular therapeutics, among others [160]. Dendritic structures make excellent delivery systems for siRNA delivery and their ability to target specific cells and to release the NA in a controlled manner without toxicity was already proven, as highlighted in this review. Reports in different areas have emerged as for example in cancer, HIV-1 infection, neurodegenerative diseases such as Familial Amyloidotic Polyneuropathy (FAP) and in stroke.

For cancer applications, siRNA delivery with dendrimers has been extensively studied in animal cancer models (Table 1). Some studies apply the dendriplexes directly to tumors [114, 115, 129, 130], others have performed systemic ad-ministration of dendriplexes functionalized with targeting moieties directed to cancer cells [101, 121], and also both approaches - intratumoral administration with cancer targeting moieties - have also been reported by Wei et al. [161]. In this last study, by using gold nanostar-based platform modified with PAMAM dendrimers, the authors proposed an

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interesting diagnosis application for tumor computed tomography imaging. Additionally, a therapeutic approach combining both photothermal and gene therapy was also proposed in vivo. Other promising dual-delivery studies using siRNA and an anticancer drug, both complexed with the dendrimer can also be found in the literature, which significantly potentiated the antitumor effect of the drug [115, 140, 142]. This can occur by sensitizing the cancer cells to the chemotherapy due to co-application of specific siRNAs responsible for regulating chemoresistance [162].

The previously mentioned study by Zhou and colleagues [113], in HIV-1 infected humanized mice, which suppressed infection after dendriplexes delivery, represents a promising strategy for the treatment of HIV.

In FAP, a hereditary disease caused by the liver expression of mutated transthyretin (TTR), Hayashi et al. used a lactosylated dendrimer (G3) conjugated with α-cyclodextrin as a novel hepatocyte-specific siRNA carrier for TTR [102, 145]. The dendriplex had a potent in vivo gene silencing effect in the liver without toxicity, constituting therefore an-other therapeutic tool for FAP treatment.

In brain ischemia studies, Kim and colleagues used arginine-modified PAMAM dendrimers (G4) to deliver siRNA against High-mobility group box 1 (HMGB1) to the post-ischemic brain with success [133]. siRNA was detected 1 h after delivery and maintained for at least 12 h and, most significantly, neuroprotection was observed.

Based on the above, the siRNA/dendrimers-based nanomedicine shows very good prospects in biomedical application of various natures, and further studies accomplished in vivo, for future translation constitutes a worth-while goal.

CONCLUSION

Among the approaches explored in gene therapy, the down-regulation of protein expression mediated by siRNAs is one of the most promising strategies. However, its success will be intimately related with the development of efficient vectors. Although there have been many advances in this field, the design of an effective and non-toxic vehicle that allows the delivery of siRNA in the desired tissue or organ needs further improvements so as to widespread the therapeutic application of RNAi. In this context, dendrimers arose as promising candidates due to their globular, well defined, very branched and multifunctionalizable structure, their low polydispersity, as well as their particular capacity to complex and protect siRNA in compact nanostructures.

Many efforts have been made to develop and optimize dendritic structures for siRNA delivery. The possibility of multifunctionalization has allowed a great variety of chemical decorations/modifications, especially the ones introduced onto the dendrimer periphery, with the aim of decreasing cytotoxicity, while improving biocompatibility. Moreover, target molecules can be linked in a controllable manner in order to get tissue-or cell specificity. Regardless of the cost and regulatory obstacles associated, the targeted approach continues being of great interest for scientists, especially for in vivo applications, since it minimizes the complicated side effects besides maximizing the site-specificity.

However, despite this great advance in the design of dendrimers to act as effective vectors of siRNA, there are some aspects/issues that still need to be improved and/or sur-passed. For instance, as previously mentioned, one important drawback is the bioaccumulation of the vector and possible subsequent toxicity after accomplishing their biological function. Because of this, more fundamental research should be directed to develop fully biodegradable dendrimers, whose goal not only concerns toxicity, but also puts forward the design of "smart" controlled delivery systems. This aims at

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triggering and/or sustaining the release of the therapeutic siRNA via the control of the vector degradation profile, as is being currently explored by us and others.

Besides optimizing the design of dendrimers for in vivo applications [163], it is important to consider also the pharmacokinetics so that a slower clearance and higher activity of the selected siRNA in the target site have to be observed. This is intimately connected to the amount and frequency of the doses that should be optimized and adjusted. Ultimately, it is obligatory to explore various safety issues in the pre-clinical scenario such as the degradability and biocompatibility. The benefits should always surpass the risks.

In the future, further contributions of dendriplex mediated siRNA delivery are expected, which may constitute a remarkable pre-clinical landmark in nanomedicine therapeutics. Combining all the aforementioned characteristics in one dendritic system would be a big step forward to translate therapeutic siRNA-dendriplexes into a clinical reality.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no con-flict of interest.

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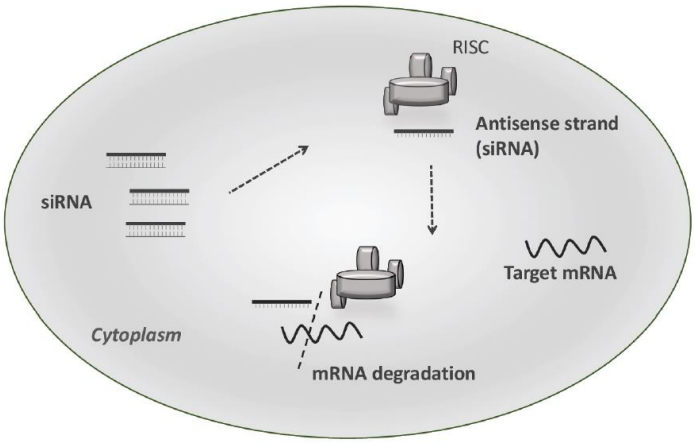

Fig. (1). RNAi mechanism of action, showing the siRNA pathway, which culminates in the downregulation of gene expression from a specific target.

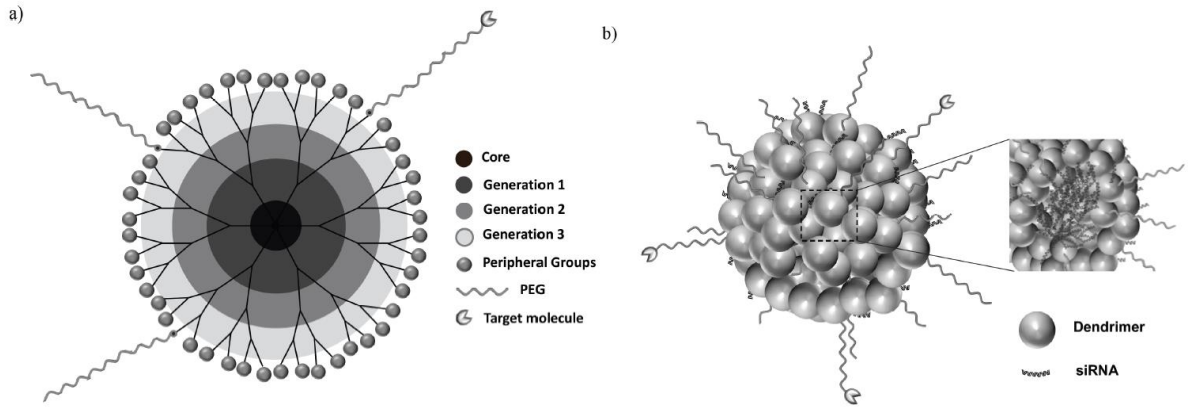

Fig. (2). Schematic representation of: **a**) a dendrimer with attached chains of poly(ethylene glycol) (PEG) and a target molecule, **b**) siRNA/dendrimer complex (dendriplex).

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Table 1. Description of distinct *in vivo* studies using dendrimers to deliver siRNA. The information is grouped according to the dendrimer type. Abbreviations: PAMAM: poly(amidoamine); PLL: Poly(L-lysine); PPI: Poly(propylene imine); PG: Poly(glycerol); TEA: Triethanolamine; PEG: Poly(ethylene glycol); i.v.: intravenous; i.n. intranasal; i.p.: intraperitoneal; Tie2: tunica interna endothelial cell kinase; HMGB1: High-mobility group box 1; HSP27: heat shock transcription factor 27; EGFR: Epidermal growth factor receptor; ATR1: Angiotensin II type 1 receptor; ApoB: Apoliprotein B, mdr1: multidrug resistance 1; PLK1: Polo-like kinase 1; BCl2: B-cell lymphoma 2; TTR: Transthyretin; Nef: Negative Regulatory Factor; D-siRNA: Dicer-substrate siRNA; PBMC: peripheral blood mononuclear cells.

Dendrimers	Generation	siRNA	Amount siRNA	Organism	Route of Administration	Time of Analysis	Organ	Main Outcome	Reference
PAMAM									
Cystamine core, PAMAM, with magnetic particles	G4	EGFR siRNA	0.5 ul/h @ 0.115 mg/ml	Mice	intracranial osmotic pump	3d / 7d	Brain tumor	Significant suppression of EGFR expression	[111]
Arginine ester of PAMAM	G4	HMGB1 siRNA	100 ng/rat	Rat	intracranial	12, 18, 24 and 48h	Brain	HMGB1 knockdown in the cortex, persistent for 48h	[133]
TEA-core PAMAMr	G5	HIV tat/rev, CD4 and TNPO3 siRNA	5x 0.25 nmol/mice. + 2x 0.25 nmol/mice	Mice	i.v.	2 w/1-20 w/ 2, 24h	Liver, blood	siRNA in PBMC and liver. Inhibition of HIV-1 infection	[113]
Amphiphilic PAMAM	G3	Hsp27 siRNA	4 x 1 mg/kg	Mice	intratumoral	6d	Tumor	Down regulation of Hsp27 and inhibition of tumor growth	[130]
PEG-modified PAMAM	G5 and G6	GFP siRNA	120 and 240 pmol	Mice	intramuscular	48h, 72h	Quadriceps muscle	Decreased GFP expression	[134]
TEA-core PAMAM	G5	Hsp27 siRNA	2x 3 mg/kg	Mice	intratumoral	1 week	Tumor	Decreased Hsp27 expression and inhibition of tumor cell proliferation	[114]
Folate-PEG-appended PAMAM with α-cyclodextrin	G3	Firefly luciferase siRNA, FITC siRNA	10 and 50 ug	Mice	intratumoral and i.v.	24h, 0-5h	Tumor, blood, liver, heart, kidney, spleen, lung	Reduced luciferase activity. FITC accumulation in kidney and tumor. No toxicity.	[101]
PAMAM in poloxamer/carbopol gel	G7	^{32}P-siRNA	2x 28 pmol/rat	Rat	i.n. and i.v.	90/180 minutes	Brain, blood	Radioactivity found in blood and olfactory bulb	[135]
Arginine ester of PAMAM	G4	HMGB1 siRNA	2ug/rat	Rat	i.n.	3, 12, 24h	Brain	HMGB1 knockdown in the amygdala, hypothalamus, cortex and striatum	[136]
Oligo-arginine conjugated PAMAM	G4	ATR1 siRNA	5 ug/kg	Rat	intramyocardial injection	3d	Heart	No increase in ATR1 after infarction and improved cardiac function	[137]
TEA-core PAMAM	G6	Akt siRNA	0.625 mg/kg	Mice	intratumoral	1 week	Tumor	Enhanced cancer cell apoptosis when Akt siRNA is administered with paclitaxel	[115]

(Table 1) contd....

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Dendrimers	Generation	siRNA	Amount siRNA	Organism	Route of Administration	Time of Analysis	Organ	Main Outcome	Reference
PAMAM									
PAMAM and PPI modified with alkyl chains	G1	Tie2 and Factor VII siRNA	1 mg/kg (Tie2) + 125 ug/kg (FVII)	Mice	i.v.	2d	Liver, blood	Nanoparticles affinity for the liver. Decreased tie2 and FVII expression.	[106]
Arginine-terminated TEA-core PAMAM	G4	Hsp27 siRNA	4x 3 mg/kg	Mice	intratumoral	2 weeks	Tumor	Effective gene silencing and decreased cancer cell proliferation	[129]
Amphiphilic PAMAM	G3	Hsp27 siRNA	10x 3 mg/kg	Mice	i.p.	5 weeks	Tumor	Down-regulation of Hsp27 and inhibition of tumor growth	[138]
TEA-core PAMAM	G5	Hsp27 D-siRNA	8x 3 mg/kg	Mice	i.p.	4 weeks	Tumor	Superior gene silencing and tumor growth inhibition.	[139]
PAMAM modified with selenium	G5	mdr1 siRNA	14x 2.5 mg/kg	Mice	intratumoral	15d	Tumor, heart, liver, spleen, lung; kidney	The dual delivery of siRNA and cisplatin enhanced the anti-tumor effect	[140]
PAMAM and PPI (modified with Epoxide-terminated alkyl chains)	G1	Tie2 siRNA	2.5, 1 and 0.5 mg/kg	Mice	i.v.	1h/2d/3d	Lung	Decreased Tie2 mRNA	[107]
Ethylenediamine core PAMAM	G5	Cy5-siRNA	Not described	Mice	i.v.	24h	Tumor, heart, liver, spleen, lung, kidney	Increased dendriplex accumulation in tumor	[141]
Folate-PEG-appended PAMAM with α-cyclodextrin	G4	siRNA PLK1	50 ug/mice	Mice	i.v.	24h, 0-20d	Blood, tumor	Improved blood circulation and RNAi effect. Suppressed tumor growth	[103]
PPI									
PPI	G5	BCL2 siRNA	1.9 mg/kg	Mice	i.v.	72h	Tumor	Preferential siRNA localization in tumor	[121]
PPI	G5	CD44 siRNA	8x 2.5 mg/kg	Mice	i.p.	48h, 0-28d	Tumor, liver, kidney, spleen, heart, lung, brain	Increased tumor apoptosis and higher activity of siRNA when using paclitaxel.	[142]
PLL dendrimers									
PLL	G6	ApoB siRNA	2.5 mg/kg, 1.5 mg/kg	Mice	i.v.	24h/24-96h	Liver, blood	Decreased ApoB expression	[143]
PLL modified with reducible spacers	Not described	ApoB siRNA	1 mg/kg	Mice	i.v.	24h, 48h	Liver, spleen, kidney, blood	Decreased Apo B in liver and blood	[144]

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Dendrimers	Generation	siRNA	Amount siRNA	Organism	Route of Administration	Time of Analysis	Organ	Main Outcome	Reference
Other Dendrimers									
Triazine	G2	Labeled siRNA, Firefly luciferase DsiRNA	35 µg/mice	Mice	i.v.	2h, 24h	Blood, lung, liver, blad-der	Dendriplexes stable in serum, siRNA presence in liver and lung	[47]
Lactosylated dendrimer conjugated with α-cyclodextrin	G3	TTR siRNA	1 mg/kg, 9 mg/kg	Mice	i.v.	48h	Liver	Decreased TTR mRNA	[145]
PG-based amphiphilic ester-bridged dendrons	G1 and G2	Non-targeting siRNA	8 mg/kg, 20 mg/kg	Mice	i.v.	1h	Serum	Cytokine levels in the serum were not elevated	[117]
Carbosilane	G2	siRNA NEF	0,54 nmol/mice	Mice	retro-orbital injection	1h, 24h	Brain, spleen, and liver	Accumulation of siRNA in brain, spleen and liver	[131]
2-(acryloyloxy)ethyl methacrylate	G1-G4	Factor VII siRNA	1, 3, 4 mg/kg	Mice	i.v.	24h, 48h, 3d	Blood, kidney, liver, spleen	High accumulation in tumor liver cells, suppression of Factor VII expression	[87]

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